PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS

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Abstract

Recombinant plasmids containing in DNA sequences coding for human preproparathyroid hormone. The invention further provides microorganisms, for example E. coli, transformed by these plasmids. Finally, the invention also provides a plasmid for insertion into yeast and a transformed yeast in which the plasmid contains DNA coding for parathyroid hormone. Parathyroid hormone is then secreted by the transformed yeast.

Description

PRODUCTION OF HUMAN PARATHYROID HORMONE
FROM MICROORGANISMS

FIELD OF THE INVENTION

This invention relates to genetically engineered microorganisms containing DNA coding for human preproparathyroid hormone.

BACKGROUND OF THE INVENTION

A number of proteins and peptides that are normally synthesized by mammalian cells have proven to have medical, agricultural and industrial utility. These proteins and peptides may be of different molecular size and have a number of different functions, for example, they may be enzymes, structural proteins, growth factors and hormones. In essence both proteins and peptides are composed of linear sequences of amino acids which-form secondary and tertiary structures that are necessary to convey the biological activity. Human parathyroid hormone has a relatively small molecular weight, which has made it possible to synthesize the peptide chemically by the sequential addition of amino acids. Thus, parathyroid hormone is commercially available, but in very small quantities at high cost.As a result, there is no human parathyroid hormone available at a reasonable price to supply the many potential medical, agricultural and industrial applications.

During the past ten years, microbiological techniques employing recombinant DNA have made it possible to use microorganisms for the production of species-different peptides. The microorganism is capable of rapid and abundant growth and can be made to -ytlsnesize the foreign product in the same manner as bacterial peptides. The utility and potential of this molecular biological approach has already been proven by microbiological production of a number of human proteins that are now available for medical and other uses.

Parathyroid hormone (PTH) is one of the most important regulators of calcium metabolism in mammals and is also related to several diseases in humans and animals, e.g. milk fever, acute hypocalsemia and otherwise pathologically altered blood calcium levels.

This hormone therefore will be important as a part of diagnostic kits and will also have potential as a therapeutic in human and veterinary medicine.

The first synthesis of DNA for human preproparathyroid hormone was described by Hendy, G.N., Kronenberg, H.M., Potts, Jr. J.T. and Rich, A., 78 Proc.

Natl. Acad. Sci. 7365-7369 (1981). DNA complementary in sequence to PTH mRNA was synthesized and made double stranded (Hendy et al., supra). This cDNA was cloned in pBR 322 DNA and E. coli 1776 was transfected. Of the colonies with correct antibiotic resistance, 23 out of 200 clones were identified as containing specific human PTH cDNA inserts. However, none of the 23 human PTH clones contained the full length insert (Hendy et al., supra). Later Breyel, E.; Morelle, G., Auf'mkolk, B., Frank, R., Blocker, H. and Mayer, H., Third European Congress on Biotechnology, 10-1-4 September 1984, Vol. 3, 363-369, described the presence of the human PTH gene in a fetal liver genomic DNA library constructed in the phage sharon 4A.A restriction enzyme fragment of the PTH gene was recloned and transfected into E. coli.

However, the work of Breyel et al., supra, demonstrated that E. coli degrades human PTH. Thus, a microoraanism which shows a stable oroduction of intact

human parathyroid hormone has so far not been described.

Further, parathyroid hormone has never before been isolated from yeast.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a plasmid containing DNA coding for human preproparathyroid hormone (HPTH) for insertion in Escherichia coli. It is another object of the present invention to provide a genetically engineered E. coli containing DNA coding for human preproparathyroid hormone.

A further object of the present invention is to provide a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. It is also an object of the present invention to provide a transformed yeast containing DNA coding for parathyroid hormone, including human parathyroid hormone, and from which transformed yeast, parathyroid hormone may be obtained.

Other objects and advantages of the present invention will become apparent as the description thereof proceeds.

In satisfaction of the 'foregoing'objects and advantages, there is provided by the present invention a novel plasmid for insertion in E. coli, containing DNA coding for human preproparathyroid hormone. The plasmid, when inserted into E. coli, functions to transform the E. coli such that the E. coli then produces multiple copies of the plasmid, and thus of the cDNA coding for human preproparathyroid hormone.

The plasmid for insertion into E. coli of the present invention and thus the transformed E. coli are distinguishable over prior art plasmids and microorganisms, for example as described in Hendy et al., supra, in that the plasmid of the present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause a production microorganism transformed with a plasmid containing this cDNA to produce preproparathyror6 hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

There is further provided by the present -invention a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. In a preferred embodiment, this plasmid is prepared by recloning the plasmid for insertion in E. coli described above.

Finally, the invention provides a yeast transformed by said plasmid for insertion in yeast such that-the yeast produces and secretes parathyroid hormone.

Thus, the invention provides a method by which parathyroid hormone may be isolated from yeast culture medium. In a preferred embodiment, the transformed yeast is Saccharomyces cervisiae. In another preferred embodiment, the parathyroid hormone is human parathyroid hormone.

Samples of pSSHPTH-10, E. coli transformed therewith, pSisLX5-HPTH1 and Saccharomyces cerevisiae transformed therewith were deposited in the American Type Culture Collection in Rockville, Maryland on September 29, 1986, under the provisions of the Budapest Treaty. The samples have been accorded the following deposit numbers:
Transformed E. coli containing pSSHPTH-10: ATCC 67223.

pSSHPTH-10: ATCC 40267.

Transformed S. cerevisiae containing pSgxLX5-HPTXI: ATCC 20821.

pSSiLX5-HPTHI: ATCC 40266.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows all possible variations of the DNA sequence coding for human preproparathyroid hormone.

Figure 2 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10.

Figure 3 shows a DNA sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

Figure 4 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10 with flanking sequences.

Figure 5 shows the actual amino acids sequence of the human preproparathyroid hormone for which the DNA sequence in clone pSSHPTH-10 codes.

Figure 6 shows the composition of the recombinant plasmid pSSHPTH-10.

Figure 7 shows a map of pALX4.

Figure 8 shows the construction of puLX5 from pL4 and pMFel-I.

Figure 9 shows the construction and schematic drawing of pSSs LX5-HPTH1.

Figure 10 shows the sequence of the MF 1-HPTH fusion gene with all possible combinations of the DNA coding for HPTH.

Figure 11 shows the sequence of the M3 I-HPTH fusion gene.

Figure 12 shows an electrophoresis plate showing the human parathyroid hormone produced and secreted by yeast and recovered from the yeast culture medium.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As indicated above, the present invention is directed to a plasmid for insertion in E. coli containing DNA coding for human preproparathyroid hormone. The invention is also directed to the resulting transformed E. coli.

The invention further is directed to a plasmid for insertion into yeast which contains DNA coding for parathyroid hormone and which is derived from the plasmid for insertion into E. coli. Finally, the invention is directed to a transformed yeast from which parathyroid hormone may be recovered.

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct size mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated in vitro from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoresis after immuno precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA -synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase.After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating the purified first strand DNA in the presence of the Klenow fragment of E. coli DNA polymerase I. The double stranded complementary DNA was made blunt ended by the action of Aspergillus bryzae single strand specific endonuclease S1 and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enzymatically added to the 3' ends of the cDNA. This modified complementary DNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector pBR 322. Resulting recombinant plasmid DNA's were transformed into E. coli Kl2 BJ 5183.Positive transformants were analysed for by colony hybridization using two different synthetic deoxyribo-oligonucleotides which covered the N-terminal coding region as well as the 3' noncoding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were identical except for some size heterogenity at the regions flanking the start codon and the XbaI site 3' for the stop codon. One clone, pSSHPTH-10, was subjected to DNA sequence analysis revealing a 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the PstI site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., supra, except for a five base pair deletion in front of the start codon.

Figure 2 shows the human preproparathyroid hormone DNA sequence of pSSHPTH-10. This may be compared with Figure 1, which shows all possible variations of the DNA sequence for human preproparathyroid hormone without the 5' double start codon. Figure 3 shows the DNA sequence of the clone of the present invention and more precisely. a portion of the DNA sequence of the plasmid for insertion into the E.Coli, coding for human preproparathyroid hormone.

with the flanking sequences.

In a preferred embodiment, the plasmid for insertion in E.C9li coding for human preproparathyroid hormone is pSSHPTH-10. the DNA sequence of which.

including the flanking sequence, is shown in Figure 4.

Figure 5 shows the DNA sequence coding for preproparathyroid hormone in pSSHPTH-10 with flanking sequences. showing the corresponding amino acid sequence of preproparathyroid hormone.

The invention further provides a plsmid for insertion into yeast containing DNA coding for parathyroid hormone.

Fig. 10 shows a partial DNA sequence for the plasmid for insertion into yeast in which: Nucleotide nos.1-173 makeup the MFs 1 promoter region and 5' noncoding sequence. 174-440 is the MFs 1 N-terminal coding sequence. 441-695 is an HPTH sequence. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10.

727-732 is from pUCl9. 733-874 is MF α 1 α 1 3' noncoding sequence and transcriptional termination signal.

The parathyroid hormone may be human or animal parahtyroid hormone, for example pig or bovine parathyroid hormone. The plasmid for insertion in yeast of the present invention may be recloned from plasmids containing DNA coding for human or animal parathyroid hormone.

Figure 11 shows theNuclectide sequence of the MFα-HPTH fusion gene from pSS4LX5-SPTHl. Nucleotide nos. 1-173 makeup the MFoG promoter region and 5' noncoding sequence. 174-440 is the MFα1 N-terminal coding sequence. 441-695 is the HPTH sequence obtained from pSSHPTE-lo. 696-726 is an SPITE 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is MFoQ 3' noncoding sequence and transcriptional termination signal.

In a preferred embodiment, the plasmid for insertion in yeast contains DNA coding for human parathyroid hormone. As shown in the following examples, the HPTH sequence from pSSHPTH-10 has been recloned and inserted in specially designed vectors for expression in Saccharomyces cerevisiae.

pSSHPTH-10 was digested to form a 288 bp BglII-XbaI fragment. This fragment was then subcloned into pUCl9 between the BamHI and XbaI sites. The subclone was then digested with Dpn I, and the largest resulting fragment was isolated. The said fragment was then digested with SalI.

The plasmid pSSsLX5-HPTHI that in yeast MAIZE cells leads to the expression and secretion of PTH was constructed in three stages:
1. Construction of the yeast shuttle vector pL4 (which replicates in both E. coli and Saccharomyces cerevisiae).

2. Cloning of a DNA fragment containing the yeast mating pheromone MFoCL gene and its insertion into the yeast shuttle vector to make the FxLX5 vector.

3. Insertion of a DNA fragment from the coding region of the HPTH gene of pSSHPTH-10 into a(LX5 in reading frame with the prepro part of the Maxi1 gene, thereby producing the vector pS9xLX5-HPTHI.

The shuttle vector pL4 was constructed by inserting into pJDB207, an EcoRI-AvaII fragment containing the ADHI promoter isolated from pADH040. A SphI fragment was then deleted, resulting in a plasmid pALXI. The PstI site in the ilactamase gene was deleted and the plasmid was partially digested with PvuI and BglI and ligated to a PvuI BglI fragment of pUC8, to form pALX2. After a further oligonucleotide insertion, the plasmid was digested with HindIrI and religated to form pALX4.

Total yeast DNA from the Y288C strain was digested with EcoRI, and the 1.6-1.8 kb fragments isolated. These were ligated to EcoRI-cleaved pBR322, and E. coli was transformed. The clones were screened for MFoa inserts by oligonucleotide hybridization.

The DNA selected thereby was then used to transform E.

coli. The resulting plasmid pMFoZ-1 was digested with EcoRI, made blunt ended by Klenow enzyme, and then digested with sglII. The MEoI fragment was isolated, and ligated to pL5 (digested with BamHI, made blunt ended with Klenow enzyme, and digested with BglII) to yield pXLX5.

In order to insert the human PTH cDNA fragment into pgLX5, the pαLX5 was digested with HindIII, creating sticky-ends and the site was made blunt ended with the DNA polymerase I Klenow fragment and dNTP.

The pαLX5 was then digested with SalI to create a sticky-ended DNA complementary to the SalI digested human PTH fragment described above.

The Sail digested human PTH fragment was then inserted into the SalI digested p > IX5. The resulting plasmid pSSLX5-PTH is shown in Figure 9.

pSSc4LX5-PTH was then inserted into yeast, thereby transforming yeast so that the yeast produces and secretes human parathyroid hormone. In a preferred embodiment, the transformed yeast is Sacchromyces cerevisiae. An electrophoresis plate showing the human parathyroid hormone from the yeast culture medium is shown at Figure 12.

Although the method fo making the plasmid for insertion in yeast by recloning pSSHPTH-10 is shown in detail, this method is shown to illustrate the invention, and the invention is not limited thereto.

The method may be applied to a variety of other plasmids containing DNA coding for human or animal PTH to produce the plasmid for insertion in yeast of the present invention.

The plasmids of the present invention and transformed microorganisms were produced as set forth in the following examples.

EXAMPLE I

Isolation of mRNA and synthesis of complementary DNA (cDNA) of human parathyroid hormone.

Starting material for the invention was parathyroid adenomas obtained from patients by surgery. The parathyroid tissue was placed on dry ice directly after removal and transported to a laboratory for preparation of RNA. The frozen tissue was homogenized with an ultra Turax homogenizer in the presence of 4 M Guanidinium thiocyanate and the RNA content was recovered by serial ethanol precipitations as described by Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., 18 Biochemistry 5294-5299 (1979). The RNA preparation was applied to oligo d(T) cellulose affinity chromatography column in order to enrich for poly(A) containing mRNA. The poly(A) rich RNA was further enriched for parathyroid hormone (PTH) mRNA sized RNA by ultracentrifugation through a 15-308 linear sucrose gradient.The resulting gradient was divided into 25 fractions and every third fraction was assayed for PTH mRNA content by in vitro translation followed by immunoprecipitation with anti PTH antiserum (Gautvik, K.M., Gautvik, V.T. and Halvorsen, J.F., Scand. J.

Clin. Lab. Invest. 43, 553-564 (1983)) and SDS-polyacrylamide gel electrophoresis (Laemmeli, U.K., 227 Nature 680 (1970)). The RNA from the fractions containing translatable PTH mRNA was recovered by ethanol precipitation.

This RNA, enriched for PTH mRNA, was used as a template for cDNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase for catalysis of the reaction (Maniatis, T., Fritsch, E.F. and Sambrook, ., Molecular Cloning pp. 230-243 (1982)). After first strand synthesis, the RNA templates were removed by alkali hydrolysis.

The second strand cDNA was synthesized by incubating the purified first strand cDNA in the presence of the Klenow fragment of E. coli DNA polymerase I (Maniatis, supra). This in vitro synthesized double stranded cDNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 (Maniatis, supra). The blunt ended double stranded cDNA was size fractionated over a 15-30% neutral sucrose gradient. The size distribution of each fraction was estimated by agarose gel electrophoresis together with known DNA fragment markers. Fractions containing cDNA larger than approximately 500 base pairs were pooled and the cDNA content was collected by ethanol precipitation.

EXAMPLE 2

Cloning of cDNA PTH in plasmid pBX 322 and transformation of E. coli K12 8J5183.

An approximate 20 base long d(C)-tail protrusion was enzymatically added to the 3 ends of the cDNA by the action of terminal deoxynucleotidyl transferase (Maniatis, supra).The(C)-tailed cDNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector

pBR322 and the resulting recombinant plasmid
DNA's were transformed into E. coli K12 BJ 5183cells which were made competent by the method of Hanahan,
D., 166 J. Mol. Biol. 557-580 (1983). A total of 33,000 transformants were analyzed for PTH cDNA content by colony hybridization (Hanahan, D. and Meselson, 10 Gene 63 (1980)).

Two to three thousand transformants were plated directly on each 82 mm diameter nitrocellulose filter, placed on top of rich medium agar plates containing tetracycline, and incubated at 37 degrees Centigrade until approximately 0.1 mm diameter colonies appeared. Duplicate replicas of each filter was obtained by serial pressing of two new filters against the original filter. The replica filters were placed on top of new tetracycline containing agar plates and incubated at 37 degrees Centigrade until approximately 0.5 mm diameter colonies appeared. The master filter with bacterial colonies was kept at 4 degrees Centigrade placed on top of the agar plate and the duplicate replica filters were removed from the agar plates and submitted to the following colony hybridization procedure.

EXAMPLE 3
Characterization of bacterial clones containing recombinant PTH cDNA and of the DNA sequence of clone pSSuPTH-10.

The cells in the respective colonies were disrupted in situ with alkali and sodium chloride leaving the DNA content of each bacterial clone exposed. The procedure allows the DNA to bind to the filter after which it was neutralized with Tris-buffer and dried at 80 degrees Centigrade. The majority of cell debris was removed by a 65 degree Centigrade wash with the detergent sodium dodecylsulphate (SDS) and sodium chloride leaving the DNA bound to the filters at the position of the former bacterial colonies. The filters were presoaked in 6xSSC (0.9M NaCl, 0.09M
Na-citrate), lx Denhart's solution (0.1 g/ml Ficoll, 0.1 g/ml polyvinyl pyrrolidone, 0.1 g/ml bovine serum albumin),
100 g/ml herring sperm DNA, 0.58 SDS and 0.05% sodium pyrophosphate for two hours at 37 degrees Centigrade (Woods, D.E., 6 Focus No. 3.

(1984)).

The hybridization was carried out at 42 degrees
Centigrade for 18 hours in a hybridization solution (6x SSC, lx Denhart's solution, 20 g/ml tRNA and 0.05% sodium pyrophosphate) supplemented with 32P-labelled
DNA probe (Woods, supra).

The DNA used as a hybridization probe was one of two different synthetic deoxyribo oligonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, et al., supra. The first probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading
TACTATGGACGTTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the restriction endonuclease XbaI located 31 nucleotides downstream of the termination codon and consisted of the nucleotide sequence CTCAAGACGAGATCTGTCACATCC.

Labelling was carried out by transfer of 32 P from 32 P-r-ATS to the 5' end of the oligonucleotides by the action of polynucleotide kinase (Maxam, A.M.

and Gilbert, W., 65 Methods Enzymol., 499 (1980)).

The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees

Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica filters. All those were picked from the original filters with the stored cDNA library and amplified for indefinitive storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a more detailed analysis by restriction endonuclease mapping, showing that all were identical except-for some size heterogenity at the regions flanking the start codon and XbaI site, respectively.

EXAMPLE 4
Clone pSSHPTH-10.

One clone, pSSHPTH-10, was subjected to DNA sequence analysis according to the method of Maxam and
Gilbert, supra. The complete structure of pSSHPTH-10 is shown in Figure 6. This clone consists of a 432 base pair long PTH cDNA sequence inserted in the Pstt site of pBR322 having 27 G/C base pairs at the 5' end and i7 G/C base pairs at the 3 end. The complete DNA sequence of the cDNA insert of pSSHPTH-10 is shown in
Figure 4. It is identical to the sequence of Hendy, et al., supra, except for a five base pair deletion
right in front of the start codon, changing the published (Hendy, et al., supra) start-stop (ATGTGAAG) signal (deletion is underlined) preceding the used start codon (ATG) to a double start signal (ATGATG).

EXAMPLE 5
Construction of the yeast shuttle vector pL4.

Before the HPTH-yeast-expression project was initiated, a family of general yeast expression vectors were developed. One of these, pL4, later was used to make pSX5-HPTH1, as described below:
The plasmid pJDB207, constructed by Beggs, J.D., nMultiple-copy yeast plasmid vectors," Von Wettstein,
D., Friis, J., Kielland-Brandt, M. and Stenderup, A.

(Eds) Molecular Genetics in Yeast (1981), Alfred
Benzon Symposium Vol. 16, 383-390, was chosen as the basis for the general expression vectors. It contains an EcoRI fragment of the yeast 2 micron DNA inserted into the pBR322 derivative pAT153. It also contains the yeast LEU2 gene. The copy number of pJDB207 in yeast cir + cells is very high relative to that of other plasmids and it is unusually stable after non-selective growth in a cir+ strain. Parent,
S.A., Fenimore, C.M., and Bostian, K.A. "Vector
Systems for the Expression, Analysis and Cloning of
DNA Sequences in S. cerevisiae," 1 Yeast 83-138 (1985); Erhart, E. and Hollenberg, C.P., "The Presence of a Defective LEU2 Gene on 2 Micron DNA Recombinant
Plasmids of Saccharomyces cerevisiae is Responsible for Curing and High Copy Number," 156 J.Bacteriol 625-635 (1983). These properties are related to a partial defective promoter in the selective marker
gene LEU2 (often named LEU2d, d for defective), Erhart et al., supra, which is not changed in the following constructs.

A 1260 base pair EcoRI-AvaII fragment containing the ADHI promoter was isolated from the plasmid pADH040. After a fill in reaction with the Klenow fragment of DNA polymerase I and all four dNTPs, BamHI linkers were attached and the fragment was cloned into

the unique BamHI site of pJDB207. From the plasmid with the promoter in a counterclockwise direction, a
1050 base pair SphI fragment was then deleted (from
the SphI site in pJDB207 to the SphI site in-the promoter fragment) leaving only a single 8amHI site.

This plasmid was designated pALXI.

The PstI site in the -lactamase gene of pALX1 then was eliminated without inactivating the gene.

pALXI was digested to completion with PstI and nuclease S1 to destroy the PstI site, and then subjected to a partial digestion with PvuI and BglI.

At the same time, a 250 base pair PvuI BglI fragment was isolated from pUC8, Vieira, J. and Messing,'J., 19
Gene 259 (1982), that contains the corresponding part of a B-iactamase gene without a PstI site. This was ligated to the partially digested pALXI. In all the ampicillin resistant clones isolated the -lactamase gene had been restored by incorporating the pUC8 fragment. This plasmid was called pALX2.

The following oligonucleotide was purchased from eros. K. Kleppe, University of Bergen, and inserted into the BamHI site of pALX2
BglII + t;;* * HindIII
GATCAGATCTGCAGGATGGATCCAAAGCTT ; initiation condon
TCTAGACGTCCTACCTAGGTTTCGAACTAG * : optimal ATG context
PstI BamHI
Plasmids with the proper orientation were isolated and designated pALX3.

Finally the pALX3 was digested with HindIII and religated to delete a HindIII fragment of 480 base pairs. The resulting vector is called pALX4. A map of pALX4 is shown in Figure 7.

pL4 is a derivative of pALX4 in which the ADHI promoter is deleted. pL4 was used as basis for the insertion of other promoters. pALX4 was first digested with BglII and SalI. The resulting sticky ends were filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, followed by religation. By this treatment, the ADHI promoter is eliminated and the BglII site regenerated to give the vector pL4.

EXAMPLE 6
Construction of piLX5.

The gene for the yeast mating pheromone MF 1 was first cloned by Kurjan, J. and Rerskowitz, I., "Structure of a Yeast Pheromone Gene (MF): A
Putative o(-Factor Precursor Contains Four Tandem
Copies of Mature o(-Factor", 30 Cell, 933-943 (1982).

The published sequence was used to reclone the MF 1 gene. Total yeast DNA from the strain Y288C was digested with EcoRI and digestion products in the size range from 1.6 to 1.8 kb were isolated from a preparative agarose gel. These were then ligated to dephosphorylated EcoRI cleaved pBR322 and used to transform E. coli By5183. The resulting clones were screened for MFI gene inserts by hybridization to a labeled oligonucleotide of the following composition:
TGGCATTGGCTGCAACTAAAGC
DNA from purified positive clones was then used to transform E. coli JA221 from which plasmid DNA was prepared. The plasmid used in the following constructs, pMFdl-I, is

shown in Figure 8.

pMSil-I was digested with EcoRI, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglII. The 1.7 kb MFoQ gene fragment was isolated from an agarose gel.

Before inserting it into the yeast shuttle vector, the Hindlit site of pL4 was eliminated by HindIII digestion, Klenow fill-in reaction and religation to give the pL5 shuttle vector. pL5 was digested with
BamHI, filled in with the Klenow fragment of DNA polymerase r and 4 dNTPs, phenol extracted and digested with BglII. After purification on gel it was ligated to the MFdl fragment to give the expression vector piLX5 as shown in Figure 8.

EXAMPLE 7

Construction of pS(LX5-HPTHI

A 288 base pair BglII XbaI fragment from the pSSHPTH-10 plasmid was isolated and subcloned in pUC19 using the BamHI and XbaI site of this vector. This subclone designated pUC-HPTH, was digested with DpnI and the largest fragment isolated. This fragment was then digested with salt and the smallest of the two resulting fragments was again isolated, yielding a sticky end on the Salt cut side and a blunt end at the DpnI cut side.

p LX5 was digested with Eindllt, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with Salt. After purication from gel, it was ligated to the HPTH fragment described above. The resulting clones had the HindIII site regenerated verifying that the reading frame was correct. This plasmid called pSSx
LX5-HPTH1 is shown in Figure 9 The sequence of the M6 I-HPTH fusion gene is shown in Figure 10.

EXAMPLE 8

Expression And Secretion Of HPTH In Yeast

The yeast strain FL200 S4, ura3, leu2) was transformed with the plasmids Lxs and pSSALX5-HPTHI using the spheroplast method. One transformant of each kind was grown up in leu medium and aliquots of the cell-free medium were analysed by SDS-PAGE developed by silver-staining (Fig. 12). Two major bands were seen in the medium from the pSSd;X5-HPTHI transformant that were not present in the medium from the piX5 transformant: one band of approximately 9000 daltons, the expected size of HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MFct-HPTH fusion product.Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

The examples are included by way of illustration, but the invention is not limited thereto. While the above examples are directed to providing a S.

cerevisiae which produces and excretes human parathyroid hormone, the method of the present invention may be applied to produce a plasmid containing DNA coding for parathyroid hormone from any species. Further, said plasmid may be inserted into any species of yeast. The invention thus is not limited to S. cerevisiae.

The cloned human parathyroid hormone produced by the yeast of the present invention has a variety of known and potential uses. For example, it is current medical theory that human parathyroid hormone will be highly effective in treating osteoporosis.

Genetically engineered parathyroid hormone may be useful in an analytical kit for measuring parathyroid hormone levels in humans and animals. Human parathyroid

hormone or fragments thereof may also be used for treatment of humans or animals displaying reduced or pathologically altered blood calcium levels. It is anticipated that many other uses will be discovered when genetically engineered parathyroid hormone is available in large quantities, for example as a result of the present invention.

The invention has been described herein with reference to certain preferred embodiments. However, as obvious variations thereon will become apparent to those skilled in the art, the invention is not to be considered limited thereto.

Claims

CLAIMS:

1. A plasmid for insertion into Escherichia coli comprising a nucleotide sequence coding for human preproparathyroid hormone having a double start codon at the 5' terminal end of said nucleotide sequence.

2. A plasmid according to Claim 1 wherein said nucleotide sequence comprises:
10 30 50
ATGATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTTY
70 90 110
YTNACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNATG
130 150 170
CAYAAYYTNGGNAARCAYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTN
190 210 230
CARGAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSN
250 270 290
CARMGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGN
310 330
GARGCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRR, wherein
M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = or G or C or T.

3. A plasmid according to claim 2 wherein the nucleotidesequence comprises:
10 30 50
TATGATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTT
70 90 110
YYTNACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNAT
130 150 170
GCAYAAYYTNGGNAARCAYYTNAASSNATGGARMGNGTNGARTGGYTNMGNAARAARYT
190 210 230
NCARGAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWS
250 270 290

NCARMGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGG
310 330 350
NGARGCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAA
370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC
430 AAAGCTCTATTA, wherein
M = A or C
R = A or G
W = A or T
S = C or T
Y = C or T
H = A or C or T
N = A or G or C or T.

4. A plasmid according to claim 2 wherein the nucleotide sequence comprises:
10 30 50
ATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTTT
70 90 110
CTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTATG
130 150 170
CATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTG
190 210 230
CAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCC
250 270 290
CAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGA
310 330 GAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGA.

5. A plasmid according to claim 3 wherein the nucleotide sequence comprises:
10 30 50
TATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTT
70 90 110
TCTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT
130 150 170
GCATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCCTAAGAAGCT
190 210 230
GCAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTC
250 270 290
CCAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGG
310 330 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAA
370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC
430
AAAGCTCTATTA.

6. A microorganism containing the plasmid of claim 1.

7. A microorganism according to claim 6 wherein said microorganism is Escherichia coli.

8. A microorganism containing the plasmid of claim 2.

9. A microorganism according to claim 8 wherein said microorganism is Escherichia coli.

10. A microorganism containing the plasmid of claim 3.

11. A microorganism according to claim 10 wherein the microorganism is Escherichia coli.

12. A microorganism containing the plasmid of claim 4.

13. A microorganism according to claim 12 wherein the microorganism is Escherichia coli.

14. A microorganism containing the plasmid of claim 5.

15. A microorganism according to claim 14 wherein the microorganism is Escherichia coli.

16. The method of making the plasmid of claim 1.

17. The method of making the microorganism of claim 6.

18. A plasmid for insertion in yeast comprising a nucleotide sequence coding for parathyroid hormone.

19. The plasmid of claim 18 wherein the parathyroid hormone is human parathyroid hormone.

20. A plasmid according to claim 19, wherein the nucleotide sequence comprises:
10 30 50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT
70 90 110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA
130 150 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT
190 210 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA
250 270 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG
310 330 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT
370 390 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG
430 450 470
ATAAAAGAGAGGCTGAAGCTWSNGTNWSNGARATHCARYTNATGCAYAAYYTNGGNAARC
490 510 530
AYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCARGAYGTNCAYAAYT
550 570 590
TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSNCARMGNCCNMGNAAR
A
610 630 650
ARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG
670 690 710
AYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAAACAGATATTGTCAGAGT
730 750 770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC
790 810 830
TTTTCATTTCTCCGTAAACAACCTGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT
850 870
CGTTACCAACTTTACACATACTTTATATAGCTAT, wherein
M = A or C
R = A or G
W = A or T
S = C or G

Y = C or T
H = A or C or T
N = A or G or C or T.

21. The plasmid of claim 20, wherein the nucleotide sequence comprises:
10 30 50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT
70 90 110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA
130 150 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT
190 210 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA
250 270 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG
310 330 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT
370 390 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG
430 450 470
ATAAAAGAGAGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCATAACCTGGGAAAAC
490 510 530
ATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT
550 570 590
TTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCCAGAGGCCCCGAAAAA
610 630 650
AGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGAGAGGCAGACAAAGCTG
670 690 710
ATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAAACAGATATTGTCAGAGT
730 750 770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC
790 810 830
TTTTCATTTCTCCGTAAACAACCTGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT
850 870 CGTTACCAACTTTACACATACTTTATATAGCTAT.

22. A microorganism containing the plasmid of claim 18.

23. A microorganism containing the plasmid of claim 19.

24. The microorganism of claim 22, wherein the microorganism is yeast.

25. The yeast of claim 24, wherein the yeast is
Sacchromyces cerevisiae.

26. The microorganism of claim 23, wherein the microorganism is yeast.

27. The yeast of claim 26, wherein the yeast is
Sacchromyces cerevisiae.

28. The method of making the plasmid of claim 18.

29. The method of making the microorganism of claim 22.